INHIBITION OF MOUSE HEPATITIS VIRUS MULTIPLICATION BY ANTISENSE OLIGONUCLEOTIDE, ANTISENSE RNA, SENSE RNA AND RIBOZYME

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ABSTRACT

Antisense nucleic acids against specific sequences of mouse hepatitis virus (MHV)-RNAs were tested for their inhibitory effects on viral multiplication in mouse DBT cells. An antisense oligonucleotide containing a sequence complementary to leader RNA was synthesized and shown to induce a significant inhibitory effect on the multiplication of MHV-JHM. A vector which expressed the antisense or sense mRNA7 of MHV was transfected into DBT cells. A decreased multiplication of MHV was observed in both cell lines. The transfected cell line which expressed ribozyme against the 5'-end of the MHV genome was established. The rate of inhibition of MHV-multiplication and the quantity of synthesized virus-specific mRNAs in this transfected cell line were the same for both antisense and sense RNA. These results show that antisense nucleic acids might be eligible for use as antiviral agents against MHV multiplication.

INTRODUCTION

It has been reported that antisense nucleic acids inhibit the expression of their target genes (Reviewed in ref. 1). Three different classes of antisense nucleic acids (antisense oligonucleotide, antisense RNA and ribozyme) are currently in use. Inside the cell, antisense nucleic acids hybridize with target RNAs and inhibit the expression of the respective target genes. Successful demonstration of antisense mechanisms for inhibition of viral replication and multiplication have been described. However, no experimental data has been reported concerning the effects of antisense nucleic acids on positive strand RNA virus except retrovirus in infected cells [1]. To inhibit MHV multiplication *in vitro* and *in vivo*, three approaches were used in this study.
[1] A 14-mer antisense oligonucleotide containing the sequence complementary to the conserved pentanucleotide sequence, UCUAA, of the leader RNA, was tested for its inhibitory effects on MHV multiplication in DBT cells. The sequence UCUAA is conserved at the initiation sites for each of the six subgenomic mRNAs and is thought to be involved in the interaction between the leader RNA and negative-strand RNA. Thus, leader RNA may take part in leader-primed transcription [2].
[2] Vectors which express sense or antisense MHV mRNA7 were transfected into DBT cells. Nucleocapsid (N) protein encoded by mRNA7 might play important roles within the transcriptional complex, since the N protein associated with MHV-specific RNAs containing the leader RNA sequences *in vitro* [3] and anti-N monoclonal antibodies inhibit viral replication *in vivo* [4].

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Furthermore, we examined the effect of sense RNA on viral multiplication because both positive- and negative-strand RNAs are synthesized in cells infected with MHV.
[3] A ribozyme designed to cleave sequences specific to MHV RNA might present a better form of antiviral agent. We used the ribozyme of the hammerhead motif[5] which was designed against the 5'-end of the MHV genome (gene1). Gene 1 is a gene important for viral transcription and replication because gene 1 is translated into proteins containing RNA-dependent RNA polymerases soon after infection[6].

MATERIALS AND METHODS

1. Cell line and MHV: Mouse astrocytoma DBT cells[7] were cultured in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS).The JHM strain of MHV[8] was used throughout this study.
2. Oligonucleotides, Plasmids and Transfection: The oligonucleotides were synthesized using the phosphoramidates method on a Beckman system Plus-1 DNA synthesizer and purified by HPLC. AL-oligo sequence is complementary to the consensus sequence, UCUAA, of the MHV leader RNA. SL-oligo is complementary to AL-oligo. GATA-oligo is a control oligonucleotide unlerated to the MHV leader RNA (Fig. 1a). A cDNA of MHV-mRNA7 was kindly provided by Dr. Siddell[9]. For RNA expression in cultured cells, cDNA of MHV were inserted into pZIP-Neo SV (x) 1[10] or pEF321-T[11]. DBT cells were transfected with these plasmids using the standard calcium phosphate precipitation procedure[12] and selected in MEM containing 1 mg/ml G418.
3. Viral infection and Plaque assay: Transfected cells and parent DBT cells were infected with MHV-JHM at a m.o.i. of 0.1 or 1.0 for 1 h at 37°C under CS-free MEM conditions. After the incubation period, cells were washed twice with CS-free MEM and fresh CS-MEM was added. After the addition of MEM with CS, plaque assays were performed to titrate infectious progeny at various times post infection (p.i.). The infectivity was expressed as plaque forming units (PFU) /ml.
4. Northern blot hybridization: Cellular RNA was prepared according to the method of Silver *et al.*[13]. The RNA samples were electrophoresed in 1% agarose gels containing formaldehyde, blotted onto nitrocellulose membranes[14] and hybridized with the ^{32}P-labeled nick-translated cDNA of MHV mRNA7 as a probe[15]. Sense and antisense probes were expressed using T7 or SP6 polymerase[14] containing ^{32}P-UTP.
5. DNA sequencing: Sequencing was carried out according to Sanger's dideoxyribonucleotide chain termination method[16].

RESULTS AND DISCUSSION

1. Inhibition of MHV multiplication by antisense oligonucleotide

The oligonucleotide sequences are shown in Fig. 1a. To investigate the effects of antisense oligonucleotide (AL-oligo) on viral multiplication, DBT cells ($5x10^4$) were incubated with MHV-JHM (0.1 m.o.i.) in the presence of oligonucleotides at concentrations ranging from 1 to 25 μM for 1 h at 37°C. During the incubation time, approximately 1.7% of the oligonucleotides was incorporated into the cells (data not shown. ref. 17). This result is in agreement with reports by Loke *et al.*[18], and Kawamura *et al.*[19] Fig.1b shows that the inhibitory effect of AL-oligo on viral multiplication was specific for the antisense sequence of the leader RNA of MHV at 5 and 10 μM. When the cells were treated with 25 μM SL-oligo, significant inhibition (20% inhibition) of viral multiplication was observed. The interaction between sense oligonucleotide (SL-oligo) with the negative-strand RNA might interfere with transcription of mRNAs by the negative-strand RNA at high concentrations of SL-oligo. To investigate the effect of AL-oligo on the synthesis of specific viral mRNAs, cellular RNA was prepared from cells infected with MHV (1.0 m.o.i.) in the presence (10 μM at 4.5 h.p.i.) or absence of the oligonucleotides and analyzed by Northern blot hybridization (Fig. 1c). The synthesis of MHV-mRNAs in the cells treated with AL-oligo was reduced, whereas no inhibitory effect on the synthesis of viral mRNA was observed in the cells treated with SL-oligo and GATA-oligo. This result suggests that AL-oligo interferes with MHV-RNA transcription at the initial stages of viral infection.

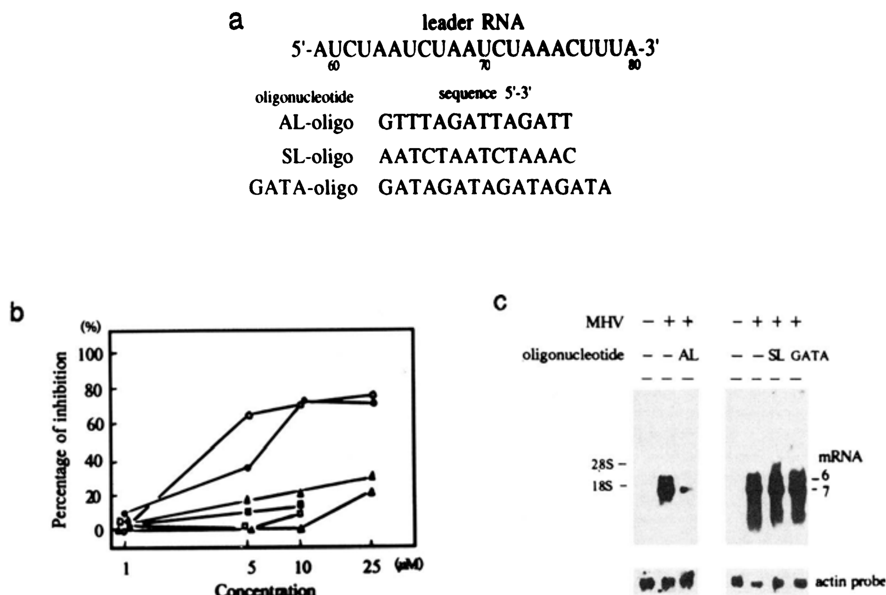

Fig. 1. (a) Sequences of oligonucleotides. (b) Effects of oligonucleotides on MHV multiplication. DBT cells were infected with MHV-JHM (0.1 m.o.i.) in the presence of AL-oligo (○, ●), SL-oligo (△, ▲) and GATA-oligo (□, ■). Plaque assays were performed at 6 (○, △, □) and 12 (●, ▲, ■) h.p.i. (c) Effects of AL-oligo on the synthesis of viral RNA. Cellular RNAs, prepared from infected (0.1 m.o.i.) or mock-infected cells treated with oligonucleotide (10 μM) at 4.5 h.p.i., were analyzed by Northern blot hybridization.

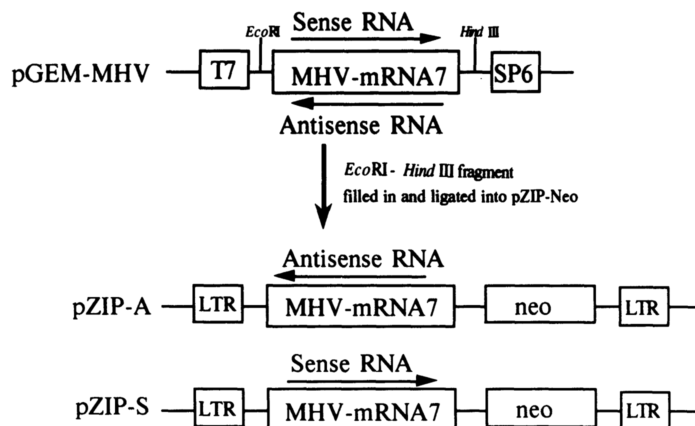

Fig. 2. Construction of plasmid pZIP-S and pZIP-A. The cDNA of MHV-mRNA7 was inserted into plasmid pGEM-1 at the *Pst* I site (pGEM-MHV). The pGEM-MHV sequence was cleaved with *Hind*III and *Eco*RI. The 1.8 kbp cDNA fragment of MHV-mRNA7 was isolated and the sticky-ends of this fragment were filled in by incubation with dNTPs in the presence of the DNA polymerase I Klenow fragments[14]. Plasmid pZIP-Neo SV (x) 1 was cleaved with *Bam*HI and filled in as explained above. The blunt-ends of both the MHV fragment and the pZIP-Neo were ligated in both sense and antisense orientation and designated pZIP-S and pZIP-A, respectively. T7 and SP6 : bacteriophage T7 and SP6 promoters , respectively ; LTR : Moloney murine leukemia virus-LTR ; neo : neomycin resistance gene.

2. Inhibition of MHV multiplication by sense or antisense RNA

To increase the level of inhibition of MHV-multiplication by antisense nucleic acids, we used sense and antisense RNA as inhibitory sequences and selected mRNA7 as a target. The treatment of infected cells with anti-N protein antibodies cause the inhibition of MHV RNA synthesis *in vitro*[20]. Therefore, the inhibition of synthesis of the N protein by antisense mRNA7 is thought to affect the MHV-transcription and/or viral multiplication. We used two vector constructions (Fig. 2), one construction being antisense RNA which is complementary to the mRNA7, and the 3'-portions of positive-strand genomic RNA and five subgenomic mRNAs. The other construction is the sense RNA which is complementary to the 5'- portions of negative-strand RNA and subgenomic negative-strand RNA. We selected negative-strand RNA as a target because some inhibitory effects could be observed at high concentrations of sense oligonucleotide (Fig. 1b).

Table I Inhibition of MHV multiplication by antisense- or sense-RNA

		9 h.p.i.		12 h.p.i.	
	cell line	PFU/ml	inhibition (%)[a)]	PFU/ml	inhibition (%)[a)]
parent DBT		2.47×10^5		1.47×10^6	
Antisense	A1	8.10×10^3	96.7	9.52×10^4	93.5
	A2	4.28×10^4	82.7	7.20×10^5	51.0
Sense	S1	1.13×10^4	95.4	1.90×10^4	98.7
	S2	3.35×10^5	——	1.60×10^6	——
	S3	7.65×10^4	69.0	3.47×10^5	76.4

a) Percentage of inhibition = $1 - \left(\frac{\text{PFU obtained from transfected cells}}{\text{PFU obtained from parent DBT cells}} \right) \times 100$ (%)

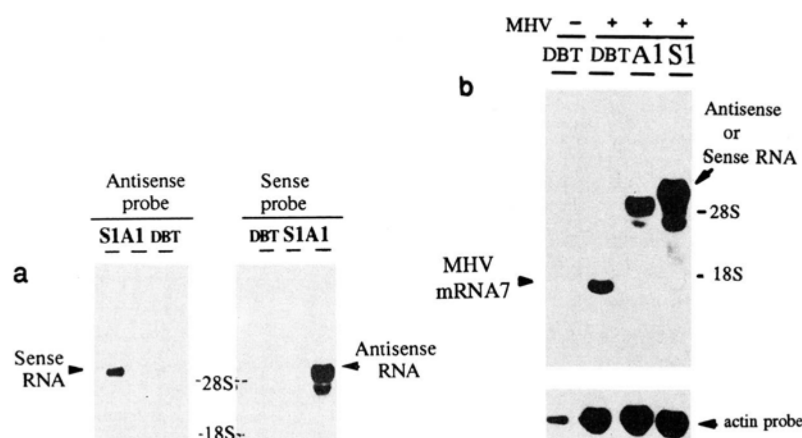

Fig. 3. (a) Single-strand RNA expression in the transfected cells. The RNAs were extracted from the S1 and A1 cells as described in the text. Samples (10 μg) were analyzed by Northern blot hybridization using single-strand ^{32}P-labeled probes. Sense or antisense probes was expressed from the pGEM-MHV sequence using T7 or SP6 polymerase, respectively (Fig. 2) and purified by Sephadex G-50 chromatography. (b) Effects of sense or antisense RNA on viral mRNA synthesis. The S1, A1 and DBT cells were infected with MHV-JHM (1.0 m.o.i.) and at 3.5 h.p.i., the cellular RNAs were analyzed by Northern blot hybridization using cDNA of MHV-mRNA7 as a probe.

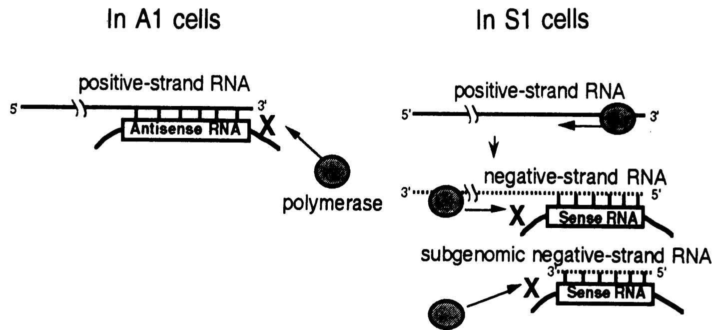

Fig. 4. Possible model for inhibition of MHVmultiplication in the S1 and A1 cells.

Furthermore, in the case of Human Immunodeficiency Virus (HIV), sense RNA inhibited viral multiplication[21]. These vectors were introduced into DBT cells and the cells were selected by culture in medium containing G418. Several transfected cell lines which constitutively expressed sense or antisense RNA were established. To investigate the effect of sense or antisense RNA on viral multiplication, plaque assays were performed. The pZIP-Neo sequences and G418 did not directly

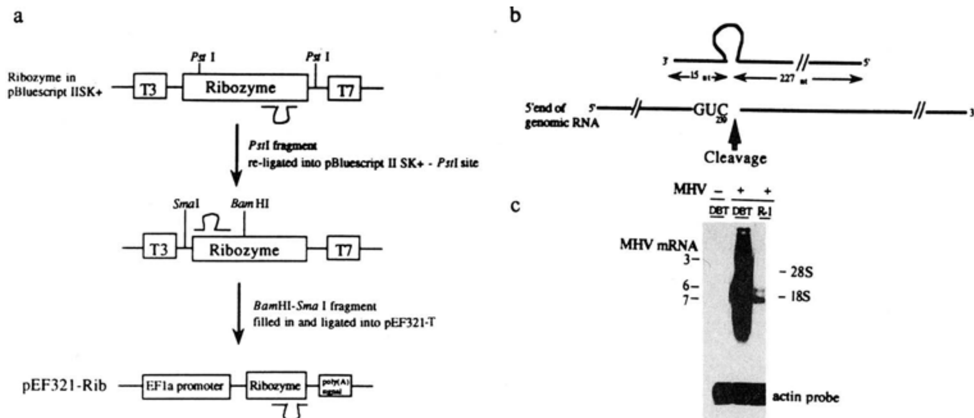

Fig. 5. (a) Construction of plasmid pEF321-Rib. Genomic RNA of MHV was extracted from MHV particles and the genomic RNA was reversed transcribed using the primer (5' GAATCCGTATACAGCATAGT 3') as forward primer and amplification by PCR was performed[14]. The temperature profile for the reaction was as follows : 95°C / 5 min then 50°C / 2 min, 72°C / 3 min, 93°C / 2 min for 30 cycles. The sequences of the forward and reverse PCR primers, (including ribozyme sequences, 5'ATGGGCAAATACGGTTTCGTCCTC ACGGACTCATCAGTCGGCTTCAAATGGG 3'), used in this reaction are based on the published sequences of the 5'-end genes of MHV-JHM[23]. The PCR reaction product was analyzed by gel electrophoresis, extracted from the gel and ligated into pBluescript II SK+ at the *Sma*I site and sequenced. This plasmid was digested with *Pst*I and re-ligated into pBluescript II SK+ at the *Pst*I site. The ribozyme fragment was digested with *Bam*HI and *Sma*I and after filling in, ligated into pEF321-T at the blunt-end (designated pEF321-Rib). This pEF321-Rib was re-sequenced for confirmation. (b) Schematic representation of ribozyme and the 5'-end of MHV genomic RNA. (c) Effect of ribozyme on MHV-RNA synthesis. DBT and transfected R-1 cells ($5x10^4$) were infected or mock-infected with MHV-JHM at 0.1 m.o.i. At 9 h.p.i., total RNA was extracted from the cells and Northern blot analysis was performed using mRNA7 cDNA as a probe.

affect the multiplication of MHV (data not shown). The multiplication of MHV in S1 and S3 cells (which express sense RNA), A1 and A2 cells (which express antisense RNA) was inhibited (Table I). MHV multiplication in A1 and S1 cells was inhibited by greater than 95% at 9 h.p.i. Northern blot analysis using single-stranded RNA probes is shown in Fig. 3a. It has been shown that interferon-mediated antiviral responses are induced by the presence of both sense and antisense RNA transcripts in infected cells[22]. The S1 and A1 cells expressed only the sense and antisense single-stranded RNA respectively, which RNAs were relatively stable in the cytoplasm (data not shown). It is thought that these RNAs are suitable candidates to hybridize with the target MHV-RNAs. On the other hand, the S2 and S3 cells expressed sense RNA at a low copy number compared with that of the S1 cells and the A2 cells expressed a longer antisense RNA as compared with that of the A1 cells (data not shown).

To investigate the effect of the sense and antisense RNA on the synthesis of viral mRNAs in the S1 and A1 cells, RNAs were prepared from cells infected with MHV at 1.0 m.o.i. and Northern blot analysis was performed. In the S1 and A1 cells, viral RNAs were hardly observed at 3.5 h.p.i. (Fig. 3b). This result suggests that the sense and antisense RNA hybridize with MHV-RNAs during the initial stages of infection. As shown in Fig. 4, it is thought that the antisense RNA of the A1 cells hybridizes with the genomic RNA during the initial stages of infection (3.5 h.p.i.). On the other hand, sense RNA which is expressed in the S1 cells, hybridizes with negative-and/or subgenomic negative-strand RNA. The MHV-RNA and antisense/sense RNA complex may delay replication of MHV in the A1/S1 cells.

3. Inhibition of MHV multiplication by ribozyme

Further investigation into the inhibition of MHV multiplication was performed using a ribozyme designed to cleave the 5'-end of the MHV genome between nucleotides (nt) 239-240 (Fig. 5b). The 5' flanking sequence of ribozyme contains 227 nt and the 3'-end contains 15 nt of a sequence complementary to MHV genomic RNA. The MHV gene 1 was cleaved at the target site with the ribozyme in cell free experiment (data not shown). This ribozyme gene sequence was cloned into pEF321-T vector and expressed in DBT cells (Fig. 5a). The so-transfected DBT cells were given the name R-1 cells and to investigate the effect of ribozyme on MHV multiplication, the R-1 cells were incubated with MHV (0.1m.o.i.). MHV-multiplication in R-1 cells was inhibited by greater than 98% at 12 h.p.i. and the synthesis of virus-specific mRNA was also reduced (Fig. 5c). These results suggest that genomic MHV RNA might be an adequate target for the study of inhibition of viral multiplication. The mechanisms and functional interactions between the ribozyme and genomic RNA have not yet been made clear and we consider this as being important to elucidate the mechanism of viral multiplication and its inhibition by ribozyme. Such a study is now in progress in our laboratory.

In this paper, we showed that antisense oligonucleotide, antisense RNA, sense RNA and ribozyme complementary to MHV-RNAs reduce viral multiplication and the synthesis of virus-specific mRNAs and that these antisense nucleic acids might find application as antiviral agents. We expect to produce transgenic mice which express antisense RNA against MHV mRNA7 to better-understand the effects of antisense RNA on viral multiplication *in vivo*.

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